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UNIVERSITY OF ALBERTA

T CELL MEDIATED IMMUNITY TO *PSEUDOMONAS AERUGINOSA* PILI

by

WALLACE DAVID SMART

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

DEPARTMENT OF IMMUNOLOGY

EDMONTON, ALBERTA

FALL 1992

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

T cell mediated immunity to

Pseudomonas aeruginosa pili

submitted by Wallace David Smart

in partial fulfilment of the requirements for the degree of

Master of Science

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Date: April 22, 1992

DEDICATION

This thesis is dedicated to my wife Lorri,

our children and my family

for their love, support, patience and understanding

throughout my long academic career.

ABSTRACT

The polar pili of *Pseudomonas aeruginosa* strains K and O each consist of a protein subunit called pilin. These molecules have been implicated in the attachment of bacteria to mucosal surfaces. The pilin subunits are each 144-residues in length and are about 49% homologous in their amino acid sequences. They have very similar biochemical properties and neither protein contains phosphate or carbohydrate residues. To delineate the T cell antigenic regions of both pilins, T cell blasts were generated from lymph nodes of PAK- or PAO-primed BALB/c mice. These blasts were tested in vitro in T cell proliferation assays for reactivity against the fragments of each pilin subunit prepared by enzymatic cleavage. Citraconylation followed by trypsin digestion (cT) cleaves both PAK and PAO pilin into four fragments, cTI (residues 1 to 30), cTII (residues 31 to 53), cTIII (residues 54 to 120), and cTIV (residues 121 to 144). T cell antigenic sites were found to reside in the cTI and cTIII regions of both pilins. Neither pilin had a T cell site located in the cTII or cTIV regions. A major T cell site was mapped to the region 82 to 110 on the PAK pilin molecule. T cell cross-reactivity between the two pilins was shown to exist in the cTI and cTIII regions. Finally, pili were found to require processing, prior to their presentation in association with class II major histocompatibility (MHC) antigens, for stimulation of pilus-immune T cells. The elucidation of T cell antigenic sites on pili is potentially useful in the development of synthetic vaccines for *Pseudomonas*-related infections in humans and animals.

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ABBREVIATIONS

APC	Antigen presenting cell
B cell	Bone marrow derived lymphocyte
BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
CPM	Counts per minute
cT	citraconylated-Tryptic digest fragment
ELISA	Enzyme linked immunosorbent assay
G.A.	Glutaraldehyde
H-2	Mouse major histocompatibility complex
HBBSS	HEPES buffered balanced salt solution
LPS	Lipopolysaccharide
MHC	Major histocompatibility antigen
Ia	I region encoded antigen
PAK	*P. aeruginosa* strain K
PAO	*P. aeruginosa* strain O
PPD	Purified protein derivative
SD	Standard deviation
T cell	Thymus derived lymphocyte

CHAPTER I

Introduction

Psuedomonas aeruginosa is a bacterium from the genus *Pseudomonas*. The pseudomonads are gram-negative, straight or curved rods with polar flagella. They are always motile and are primarily found in the soil and water. On one hand, the pseudomonads are ecologically important organisms. They are probably responsible for the degradation of many soluble compounds derived from the breakdown of plant and animal materials. In addition, they are involved in the nitrogen cycle in nature by acting as denitrifiers. On the other hand, a few of the pseudomonads are pathogenic, causing a wide variety of diseases in plants, animals and humans. However, since the pseudomonads can be readily isolated from the soil and perform important ecological roles, they can not be considered obligate parasites. As pathogens the pseudomonads, including *P. aeruginosa*, are primarily opportunistic, initiating infections in hosts whose resistance is low.

A. *Pseudomonas* infections

In humans *P. aeruginosa* is a opportunistic pathogen. It is a leading cause of morbidity and mortality in individuals with illnesses that either involve a disruption of the natural barriers that protect against infection, or a disturbance in normal host defense mechanisms. Individuals with a disruption of their natural

barriers include those with burn or corneal injuries, those patients intubated in intensive care units, those with tracheostomies and those receiving chronic intravenous therapy. Individuals who have a disturbance in normal host defense mechanisms include those with metabolic, hematologic and malignant diseases; as well as those patients receiving treatment with immunosuppressive agents, corticosteroids, antibiotics or radiation. In particular, it causes significant morbidity and mortality in individuals with cystic fibrosis, as described below.

As can be appreciated, individuals with the kinds of conditions listed above, are often hospitalized. *P. aeruginosa* is an important nosocomial pathogen. The organism is frequently found in moist areas such as sinks, antiseptic solutions, and washrooms. It is often passed from patient to patient on the hands of hospital personnel. *P. aeruginosa* is the most common pathogen recovered from patients who have been hospitalized for more than a week. The likelihood of developing a *P. aeruginosa* infection is much greater among colonized patients and the longer a patient remains in hospital, the greater the chance of becoming colonized (Bodey *et al.*, 1983). Presently, *P. aeruginosa* is estimated to cause between 10-20% of all infections in most hospitals (Bodey *et al.*, 1983). In immunocompromised individuals, a *P. aeruginosa* infection can be life threatening.

1. Cystic Fibrosis

Cystic fibrosis is an autosomal recessive disease affecting the exocrine glands of the body. It usually begins early in infancy, and individuals with this

illness rarely live beyond their late twenties or early thirties. It is characterised by viscid mucous secretions, pancreatic insufficiency and chronic respiratory infection. Primarily affected are the digestive and respiratory systems. It is thought that the lungs are normal at birth. The small airways then become obstructed due to the abnormally thick secretions. The disease then progresses to involve a superimposed bacterial infection and finally chronic bronchitis and bronchiectasis. While many different organisms are involved early on, the organism involved in the chronic infective process is usually *P. aeruginosa*. As a result, infections with *P. aeruginosa* cause a considerable amount of morbidity and mortality in these individuals. These patients are known to have an incidence of 60% of chronic purulent *Pseudomonas* bronchitis, which is one of the leading causes of death (Shwachman *et al.*, 1977). Although the gene for cystic fibrosis has been identified (Tsui *et al.*, 1985, Wainwright *et al.*, 1985) which will allow for the development of improved treatments, a better understanding of the pathogenesis of *P. aeruginosa* may also lead to improved treatments in individuals with this illness.

B. *Pseudomonas* virulence

The pathogenicity or virulence of an organism is its ability to cause disease. The more virulent an organism, the easier it is for the organism to cause disease. In addition, the disease it causes is usually of a more severe type. Smith (1984) has described a number of steps that must occur in order for an organism to be pathogenic. First, it must adhere to and colonize the mucosal surfaces. Second,

it must penetrate the mucosal surfaces. Third, it must establish itself and multiply in the hosts's tissue. Fourth, it must be able to resist or interfere with the host's defense system that is trying to destroy it. Finally, it must cause damage to the host's tissue.

Most organisms possess unique characteristics or factors that aid the organism in achieving the above steps. *P. aeruginosa* is no exception in that it possesses a number of factors which can increase and aid in its virulence. These factors can be divided into those that are secreted exoproducts and those that are components associated with the cell surface. The virulence factors that are secreted include: exotoxin A (Iglewski *et al.*, 1977), exoenzyme S (Iglewski *et al.*, 1978), various proteases such as elastase and alkaline phosphatase (Morihara *et al.*, 1964), and hemolysins such as phospholipase C (Johnson *et al.,* 1980; Berk *et al.*, 1987). All of these factors help *P. aeruginosa* resist or interfere with the host's defence system by destroying or degrading various substrates. Some of the virulence factors associated with the cell surface include lipopolysaccharide (LPS) (Pollack *et al.*, 1979), alginate (or exopolysaccharide) (Woods *et al.*, 1985) and pili (Woods *et al.*, 1980). In our laboratory, we have been concerned with the role of pili in pathogenesis.

C. *Pseudomonas* pilus - structure

The pili of *P. aeruginosa* are flexible filamentous structures consisting of a polymer of a single protein subunit called pilin. These pili are chromosomally

encoded and protrude from the ends of the rod-shaped organisms. They have diameters of 5.4 nm and average lengths of 2,500 nm (Weiss *et al.*, 1971; Bradley *et al*, 1972). The pilin subunits of each pilus have molecular weights of 15,000 and are arranged in a helix of five subunits per turn, with each turn having a 4.1-nm pitch (Watts *et al.*, 1983a). Although serologically unrelated, the two types of *P. aeruginosa* pili (those in strains PAK and PAO) have very similar biochemical properties (Paranchych, *et al.*, 1979). The amino acid sequence compositions of the two *P. aeruginosa* pilin subunits are similar but not identical, and neither protein contains phosphate or carbohydrate residues (Paranchych *et al.*, 1979; Sastry *et al.*, 1985a). *Pseudomonas* pili belong to a class of pili which are present in a wide range of gram-negative bacteria including *Neisseria gonorrhoeae, Neisseria meningitidis, Moraxella bovis, Moraxella nonliquefaciens,* and *Bacteroides nodosus* (Elleman *et al.*, 1984; Froholm *et al.*, 1977; Marrs *et al.*, 1985; Paranchych *et al.*, 1978; Perry *et al.*, 1987; Schoolnik *et al.*, 1984). These pili are characterized by an unusual residue at the amino terminus, *N*-methylphenylalanine, followed by a highly conserved sequence of about 28 hydrophobic residues. They are generally referred to as NMePhe pili (Pasloske *et al.*, 1988).

Pili seem to perform three distinct functions. Primarily, they are involved in attachment and adherence of the organism to mucosal surfaces (Woods *et al.*, 1980; Doig *et al.*, 1988). They are also involved in a type of motility called twitching motility, which can be observed on solid agar surfaces (Henrichsen, 1983). Finally,

they can be used as receptors by a number of bacteriophage (Bradley *et al.*, 1974). They are not, however, involved in bacterial conjugation.

D. Mechanisms of infection

The first stage in the establishment of an infection by *P. aeruginosa* is attachment to the mucosal surface. In individuals with a disruption of the natural mucosal surfaces (ie burn victims) or those with a defect in host defenses (ie immunocompromised patients) epithelial cell adhesion is perhaps easier. *P. aeruginosa* can use both pili and alginate (the principle component of the *P. aeruginosa* capsule) as adhesins to mediate attachment to human epithelial cells (Doig *et al.*, 1987, 1988; Ramphal and Pier, 1985; and Woods *et al.*, 1980). Since pilus adhesion is more effective than alginate adhesion, it probably mediates the initial adherence of *P. aeruginosa* to the epithelium (Doig *et al.*, 1987, 1988; McEachran and Irvin, 1986). It has been established that successful colonization of *P. aeruginosa* on the mucosal surface is dependent on the presence of pili (Woods *et al.*, 1980). Piliated bacteria that adhere to mammalian cells are often more pathogenic than their non-piliated counterparts because the pili enable them to become anchored to the host tissue and resist elimination by body fluids. Recently, Doig *et al.*, (1988) and Irvin *et al.*, (1989) have characterized the region on the PAK pilin subunit that binds to human epithelial cells.

Successful adherence to the epithelial cell surface allows the next stages of the infective process to proceed which are colonization and subsequently

infection. For example, in cystic fibrotic patients, colonization of the respiratory mucosal surface is followed by a descending-infection like mechanism to produce the chronic pulmonary infections (Rivera *et al.*, 1982; Speert, 1985).

E. Treatment of *P. aeruginosa* infections

1. Antibiotics

Antibiotic treatment is available for most *P. aeruginosa* infections. However, *P. aeruginosa* has a low susceptibility to antibiotics, and, as a result, a combined chemotherapeutic approach is often required. The most common combination consists of an aminoglycoside and an extended spectrum penicillin. Most recently, newer antibiotics such as the quinolones have also proved to be effective in treating *P. aeruginosa* infections. These newer antibiotics have the added advantage of being less toxic for the patients. However, because of the high resistance of *P. aeruginosa* to antibiotics and the debilitated condition of the individuals who acquire a *P. aeruginosa* infection, it is never possible to completely eradicate *P. aeruginosa* from these individuals and patient mortality still remains high. Thus, other modes of combating *P. aeruginosa* infections are required.

2. Immunoprophylatic approach

Mammalian hosts are capable of providing at least three lines of defense towards *P. aeruginosa* infections. Immediate protection is provided by macrophages and polymorphonuclear leucocytes (PMN's), while B cell-mediated

humoral immunity and T cell-mediated cellular immunity are slower responses to organisms that survive the initial phagocytic processes.

Since colonization is required for infection, and adherence is required for successful colonization, if adherence was prevented, then so might colonization and the ability of the organism to produce an infection. Furthermore, since pili are probably the mediators of attachment and adhesion, an understanding of their immunologic properties is potentially useful in identifying regions on the pilus molecule that may be useful in the development of synthetic vaccines. Such vaccines would be targeted to elicit B or T cell responses in hosts against pili.

In addition, once developed, these synthetic vaccines could be used to immunize those individuals at highest risk for a *P. aeruginosa* infection, such as cystic fibrosis patients. Conversely, in those individuals where active immunization is not possible (ie those with an ongoing *P. aeruginosa* infection), pooled gamma globulin, obtained from voluntary donors immunized with the synthetic vaccine, could be administered passively as a way of aiding the fight against the organism. The development of such a synthetic vaccine would involve an understanding of both the B and T cell antigenic sites on the pilus molecule.

i) B cell studies with pili

Studies regarding the host defence mechanisms against *P. aeruginosa* have concentrated on the role of B cell or antibody-mediated protection. Antibodies to a number of *Pseudomonas* antigens have been identified in patients with

Pseudomonas infections (Klinger *et al.*, 1978; Pollack *et al.*, 1979). In addition, a few studies have been performed to elucidate the B cell antigenic properties of pili. Watts *et al.* (1983b) raised polyclonal antibodies against *P. aeruginosa* PAK pili in order to determine which regions of the pilin were responsible for B cell antigenicity. Enzymatically cleaved fragments of the pilin were used, and four regions of strain PAK pilin were shown to be B cell antigenic sites in direct enzyme-linked immunosorbent assays (ELISAs) in which an anti-PAK pilus polyclonal serum was used. Sastry *et al.* (1985b), using a competition ELISA protocol, showed that the major B cell antigenic site was located in a region from residues 82-110 on the PAK pilin molecule.

When anti-PAO pilus antiserum was assayed for cross-reactivity with strain PAK pilin fragments, only the N-terminal region of the molecule was positive (Watts *et al.* 1983b). However, Lee *et al.* (1989) using antipeptide antibodies, rather than antipili antibodies, raised against the synthetic strain PAK region 128-144 peptide (intact disulphide bridge), showed that these antibodies were able to bind strain PAO pili in direct ELISAs. Thus, they showed that while antiserum to the complete protein may not be able to give rise to cross-reacting antibodies in the C-terminal region, it is possible to raise cross-reacting antibodies using synthetic peptides as immunogens. They went on further to demonstrate that by using antibodies raised against synthetic peptides from both strains PAK and PAO pili, that cross-reactive and strain specific B-cell antigenic sites could be identified (Lee *et al.*, 1990).

ii) T cell studies in *P. aeruginosa* infections

T cells play a very crucial role in the induction of humoral and cell mediated immune responses. Their role as effector cells in any immune response by an organism makes the understanding of their activation mechanisms critical. T cells respond to antigens when the latter are presented in the context of self-major histocompatibility complex (MHC) antigens. These MHC antigens are located on the surface of most cells in the body including macrophages, which are the cells involved in the first line of defense against a *Pseudomonas* infection. The *P. aeruginosa* cells are phagocytosed by the macrophages, processed (digested and degraded into various components) and presented to T cells. This presentation (antigen + self-MHC), along with the release of immunomodulators by the macrophages, activates the T cells. Once activated the T cells initiate further responses by the humoral and cellular arms of the immune system. As can be seen, a complete understanding of the T cell response to *P. aeruginosa* is important, over and above an understanding of the B cell responses.

In studies involving the role of T cells in *Pseudomonas* immunity, it has been demonstrated that this bacterium is capable of eliciting T cell responses in a number of species (Markham *et al.*, 1984; Markham *et al.*, 1985; Parmely *et al.*, 1984; Parmely and Horvat, 1986; Pier and Markham, 1982; Porwell *et al.*, 1983; Powderly *et al.*, 1986). Sorensen *et al.* (1977) originally reported that lymphocytes from patients with cystic fibrosis who have advanced pulmonary disease have low responses to *P. aeruginosa* in vitro. This hyporesponsiveness is both acquired and

specific and may play an important role in the irreversibility and destructiveness of the *Pseudomonas* infection.

Later Garzelli *et al.* (1980) and Campa *et al.* (1982) reported that mice with repeated systemic infections caused by *P. aeruginosa* can induce a specific delayed-type hypersensitivity after a challenge in the foot-pad. Furthermore, T lymphocyte-enriched spleen cells from sensitized donors can specifically transfer this delayed-type hypersensitivity to syngeneic recipients. However, the T lymphocyte-enriched spleen cells can not transfer protection against a lethal challenge. This result indicates that in systemic *P. aeruginosa* infections, a dissociation may exist between delayed-type hypersensitivity and acquired cellular resistance.

In other studies, Pier and Markham *et al.* (1982) demonstrated, in a murine model, that Thy-1$^+$ spleen cells from *Pseudomonas*-immune donors have the ability to transfer the protection against a bacterial challenge. They used a high-molecular-weight polysaccharide that was isolated from *P. aeruginosa* culture supernatants and the cytotoxic drug vinblastine, as an antigenic system. Results of further studies by these investigators also suggested a more direct role for T cells in providing protection against *P. aeruginosa.* They describe a Lyt-1$^-$, 2,3$^+$, I-J$^+$ T cell as the final effector cell in the in vitro killing of this extracellular bacterium, and that the T cell killing is mediated by the lymphokine interleukin 1. (Markham *et al.,* 1984; Markham *et al.,* 1985). This was the first indication that T cells may be involved in a role other than that of a helper cell for antibody production in the

immunity against *Pseudomonas*.

Results of recent studies in patients with cystic fibrosis by Parmely *et al.* (1984) and Parmely and Horvat (1986) have identified two secreted T cell-stimulating antigens from *P. aeruginosa*. While responses were directed against both elastase and alkaline phosphatase, the majority of the response was towards the latter. Results of these studies provide an unique antigenic system to study the immunity to *Pseudomonas*.

Prior to the work described in this thesis, the T cell antigenic sites on the pilus molecule had not been determined. An understanding of the T cell antigenic sites on the pilus molecule may help in the production of synthetic vaccines or synthetic peptides which may be able to modulate the immune response to the *P. aeruginosa* organism.

F. Aims of the project

Since pili are important in the attachment of *P. aeruginosa*, the T cell responses to pili in mice were investigated for the first time. Polyclonal T cell populations, from the lymph nodes of mice, immunized against *P. aeruginosa* strains K and O pili, were used to determine the T cell recognition sites on the pilin molecules.

Chapter two describes the recognition of PAK pilin by PAK-immune T cell blasts. Two antigenic sites are described. One is located in the cTI region of the PAK pilin molecule (residues 1 to 30). The other site is located in the cTIII region

(residues 54 to 120) and more specifically mapped to residues 82 to 110. In addition, a cross-reactivity between PAK and PAO pilin is described at the T cell level. This cross-reactivity was postulated to be due to the sequence homology between the two pilins in the cTI region.

Chapter three primarily describes the recognition of PAO pilin by PAO-immune T cell blasts. Like the results for PAK pilin, two T cell antigenic sites, in the same regions as described for PAK pilin, were identified. Comparisons were made between PAK and PAO pilin in regards to their B and T cell antigenic sites. In addition, further evidence for T cell cross-reactivity existing in the cTIII region, as well as the cTI region is given. Finally, the need for the presence of class II major histocompatibility (MHC) antigens and the need for processing of the pili, prior to its expression on the surface of antigen presenting cells, is described.

Chapter four offers general discussion and future prospects.

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CHAPTER II
Mapping of the T cell recognition sites of *Pseudomonas aeruginosa* PAK Polar Pili[1]

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen and is a leading cause of morbidity and mortality in immunocompromised patients, such as individuals with cystic fibrosis, thermal injury, and leukemia (Bodey *et al.*, 1983). In all these individuals, there is a destruction of the natural mucosal barrier coupled with the ability of this organism to adhere to the mucosal surface. It has been established that successful colonization of the organism on the mucosal surface is dependent on the presence of pili (Woods *et al.*, 1980). Piliated bacteria that adhere to mammalian cells are often more pathogenic than their nonpiliated counterparts because the pili enable them to become anchored to the host tissue and resist elimination by body fluids.

P. aeruginosa PAK and PAO produce chromosomally encoded polar pili which are flexible filaments with diameters of 5.4 nm and average lengths of 2,500 nm (Bradley, 1972; Weiss, 1971). Each pilus consists of pilin subunits with molecular weights of 15,000 arranged in a helix of five subunits per turn, with each turn having a 4.1-nm pitch (Watts *et al.*, 1983a). Although serologically unrelated, the two types of *P. aeruginosa* pili (those in strains PAK and PAO) have very

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similar biochemical properties (Paranchych, *et al.,* 1979). The amino acid sequence compositions of the two *P. aeruginosa* pilin subunits are similar but not identical, and neither protein contains phosphate or carbohydrate residues (Paranchych *et al.,* 1979; Sastry *et al.,* 1985a). In addition, these two pilin subunits share extensive amino-terminal amino acid sequence homology with the pilin subunits from *Moraxella nonliquefaciens* (Froholm and Sletten, 1977), *Moraxella bovis* (Marrs *et al.,* 1985), *Neisseria gonorrhoea* (Schoolnik *et al.,* 1983) and *Bacteroides nodosus* (McKern *et al.,* 1983).

In previous work, polyclonal antibodies raised against *P. aeruginosa* pili were used to determine which regions of the pilin were responsible for the antigenicity. Four proteolytic fragments of PAK pilin, which contain sequential antigenic determinants, were identified (Watts *et al.,* 1983b), and a major antigenic determinant was delineated in a region of the protein encompassing residues 82-110 (Sastry *et al.,* 1985b). In the present study, T cell responses induced by the pili in mice were investigated for the first time. Polyclonal T cell populations, from the lymph nodes of mice, raised against *P. aeruginosa* pili were used to determine the T cell recognition sites on the pilin. Various subfragments of the pilin molecule were prepared by enzymatic digestion. The reactive sites were then compared with those that bound anti pilus antisera, for identification of any similarities or differences. It is of considerable interest to delineate the antigenic determinants of the pilus subunit. Precise information relating to pilus antigenic sites is potentially useful in the development of synthetic vaccines for *Pseudomonas*-related infections

in humans and animals. Furthermore, since T cells are the major regulatory cells in the immune system, these studies are potentially useful in identifying peptides which could be tested for their ability to regulate the immune response against piliated *Pseudomonas* strains.

MATERIALS AND METHODS

Bacteria. The *P. aeruginosa* strains used were PAK/2Pfs and PAO/DB2. Both are multipiliated mutants which were originally obtained from D. E. Bradley, Faculty of Medicine, Memorial University, St. John's, Newfoundland, Canada, and have been described previously (Frost and Paranchych, 1977; Paranchych *et al.*, 1979).

Pilus purification. PAK and PAO pili were purified as described previously (Paranchych *et al.*, 1979).

Antigens. The preparation of the cT peptides (abbreviated cT for citraconylation followed by trypsin digestion) was described previously (Watts *et al.*, 1983b). In brief, modification of the 12 lysines in pilin was achieved with citraconic anhyride, and this was followed by trypsin digestion of the pilin. This achieves arginine-specific cleavage of the pilin and yielded four convenient fragments, cTI (residues 1 to 30), cTII (residues 31 to 53), cTIII (residues 54 to 120), and cTIV (residues 121 to 144). Initially, the digestion yields both a soluble and an insoluble fraction after centrifugation of the digest. The cT peptides in the soluble fraction (cTII, cTIII, cTIV) were purified by column chromatography on a Sephadex G-50 column and the purity was checked with amino acid analysis, high-voltage paper electrophoresis, N-terminal analysis and sodium dodecyl sulfate-polyacrylamide gels (Watts *et al.*, 1983b). The insoluble pellet primarily contains the cTI peptide.

This fraction was further purified in the presence of octyl glucoside on a Sephadex G-75 column. The eluate was then dialysed to remove the detergent and purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Watts *et al.,* 1983b). The lysine residues, initially blocked with citraconic anhydride, were deblocked prior to the use of the peptides as immunogens or in assays.

Preparation of cTIII subfragments. In addition to tryptic digestion of the citraconylated protein to generate the cT fragments as described above, cyanogen bromide (CB digestion was used to generate the fragments CBI (residues 1 to 7), CBII (residues 8 to 104), and CBIII (residues 105 to 144). Then, tryptic (T) cleavages were performed on the decitraconylated cT and CB peptides to produce the subfragments used in these experiments. The methods for generation and purification of these subfragments has been described previously (Watts *et al.,* 1983b). The subfragments were designated according to the procedure used to generate them, and include cTIII-T_4 (residues 70 to 81), cTIII-T_1 (residues 82 to 110), cTIII-T_1-CBII (residues 82 to 104), $Th_3$8 (residues 104 to 114) and 67-87 (residues 67 to 87) which was synthesized by Dr. R.S. Hodges, Department of Biochemistry, University of Alberta.

Animals. Mice of strains BALB/c, C57B1/10J and CBA/J were bred at the Ellerslie Animal Farm of the University of Alberta, or purchased from the Jackson

Laboratory, Bar Harbor, Maine.

Immunizations. Whole bacteria immunization were performed by mixing a suspended saline solution of viable bacteria (2×10^8/ml) with an equal volume of Complete Freund's Adjuvant (CFA), and injecting this suspension (50 µl/10^7 bacteria/mouse) into the hind foot pads of mice. Purified pili and peptide immunizations were performed by injecting 10-25 µg of purified pili or peptide, contained in either CFA or saline, into the hind foot pads.

T cell proliferation assays. These were performed essentially as described earlier (Fotedar *et al.*, 1985). In brief, mice were immunized with 10-25 µg of antigen in equal volumes of CFA per hind foot pad. Popliteal lymph nodes were excised and nylon wool purified lymph node T cells (5×10^5 to 10^6 cells) were incubated, in triplicate, with appropriate doses of antigen and irradiated (3000 rads) syngeneic spleen cells (10^6 cells) for four days in culture medium (RPMI-1640 containing HEPES, glutamine, 5×10^{-5} M 2-mercaptoethanol, 10% heat-inactivated fetal calf serum and Penicillin-Streptomycin). Incorporation of [^{3}H]thymidine, by pulsing with 1 µCi/well for the final 24 hours, was used as an index of proliferation. Results are expressed as counts per minute (CPM) incorporated or as stimulation index.

RESULTS

Ability of mice to produce a T cell response against pili. We tested the ability of BALB/c mice to produce a T cell response against pili in two ways. First, mice were immunized with 10^7 CFA-immobilized bacteria in the foot-pad. Popliteal lymph nodes were removed 10-15 days later to test for a proliferative response against purified pili. Second, mice were immunized in the foot-pad with purified pili either in CFA or in saline, and 10-15 days later lymph nodes were removed for a T cell proliferation assay. The results (Table 2.1) indicate that mice do produce a T cell response against purified pilin when immunized with either whole bacteria or pilin itself. A good response was observed, with a maximum response occurring at the level of 40μg of added antigen per well (approximately 150-200μg/ml). As expected, the response generated by immunization with the purified pili was greater than that obtained by immunization with whole bacteria. This is probably due to the presence of other strong immunogens on the bacteria, such as lipopolysaccharide and outer membrane proteins. It also reflects the presence of fewer pili molecules, per injection, on whole bacteria than when a purified pili preparation is used as an immunogen. The response to the pili was greater when the pili were immunized in CFA rather than in saline and there was no observable response to an irrelevant antigen (data not shown).

Response of various mouse strains to *P. aeruginosa* PAK pili. To determine whether there was a variation in the ability of different mouse strains to respond

to *P. aeruginosa* PAK pili, we tested the responses of three standard mouse strains to purified pilin. The results (Figure 2.1) indicate that all three strains, BALB/c, C57Bl/10 and CBA/J, can produce a T cell response to the PAK pili. The response of all the mouse strains was about equal. Further studies were undertaken using the BALB/c mouse strain because of its good response and availability.

Ability of BALB/c mice to response to both types of *P. aeruginosa* pili. The ability of BALB/c mice to respond to both types of *P. aeruginosa* pili, PAK and PAO, was investigated by immunizing two groups of mice, one with PAK pili and the other with PAO pili. The resulting T cell blasts were tested for their responsiveness against both types of pili. There was significant cross-reactivity in both directions (Figure 2.2), with the response of the T cell blasts being the greatest when they were stimulated by the immunogen by which they were induced.

Response of T cell blasts to various proteolytic fragments of PAK pilin. There are three arginine residues at positions 30, 53, and 120 in the pilin molecule. This conveniently divides the protein into four regions. Therefore, arginine-specific cleavage, as described above. was used to generate the four fragments cTI (residues 1 to 30), cTII (residues 31 to 53), cTIII (residues 54 to 120), and cTIV (residues 121 to 144). The peptide cTI, which encompasses the extremely

hydrophobic N terminus of pilin, was largely found in the insoluble pellet that was obtained after centrifugation of the digest. This material could be solubilized in the detergent octyl glucoside and was further purified on Sephadex G-75 column in the presence of octyl glucoside, as described previously (Watts *et al.*, 1983b). The major peak obtained on this Sephadex G-75 eluted at an apparent molecular weight of 33,000 and consisted of aggregated cTI plus bound detergent. This detergent was removed from the aggregate by dialysis; and the peptide checked for purity by using sodium dodecyl sulfate-polyacrylamide gels, before it was used to immunize mice.

The remaining cT peptides (cTII, cTIII, and cTIV) were contained in the soluble portion of the initial cT digest and were purified as described above. The ability of PAK-immune T cell blasts to respond to these three soluble cT peptides was investigated, and the results are given in Table 2.2. The only peptide to elicit a response from the PAK-immune T cell blasts was cTIII. Neither cTII nor cTIV was able to stimulate the blasts to proliferate.

We were not able to test the response of PAK pilus-immune T cell blasts against the fragment cTI because of the insolubility of this peptide. Instead, to determine whether an antigenic site existed within cTI, this peptide was used to immunize mice; and the resulting cTI-specific T cell blasts were tested for their ability to be stimulated by PAK pili. As the results in Figure 2.3 indicate, both PAK and PAO pili were able to stimulate the cTI-immune T cells to proliferate, whereas an irrelevant antigen was not.

We also tested the ability of the cTII, cTII, and cTIV peptides to produce antigen-specific T cell blasts. Only the cTII peptide was able to induce the production of antigen-specific T cell blasts. The cTII and cTIV peptides did not produce any detectable T cell proliferative response in BALB/c mice (data not shown).

Mapping of the antigenic determinant within the cTIII region. As described above, only the cTIII peptide was able to stimulate PAK-immune T cells to proliferate. Since cTIII represents a large portion of the pilin molecule (residues 54-120), investigations were undertaken to localize antigenic sites within this region by testing smaller subfragments of cTIII (see above). For these experiments, two approaches were used. First, mice were immunized with the purified pili, and second, mice were immunized with the cTIII fragment itself. Detectable levels of response were observed with the subfragments cTIII-T_1 (residues 82-110) and cTIII-T_1-CBII (residues 82-104), when mice were immunized with the purified pilin (data not shown). The response to these subfragments was greater when mice were immunized with the cTIII peptide instead. Presumably, this was due to the fact that a larger number of clones were generated towards these sites in a cTIII immunization, as opposed to those generated in a whole-pilin immunization. There was no response to the peptides cTII-T_4 (residues 70-81), $Th_3$8 (residues 104-114) or the synthetic peptide 67-87 (residues 67-87). The results are shown in Figure 2.4 and summarized in Figure 2.5.

DISCUSSION

Pseudomonas aeruginosa is an extracellular, gram-negative bacillus and, as stated above, is a leading cause of morbidity and mortality in individuals with a disruption of the natural barriers that protect against infection, or those who have a disturbance in normal host defence mechanisms. Presently, *P. aeruginosa* is estimated to cause between 10% and 20% of all infections in most hospitals (Bodey *et al.*, 1983). It is the most common pathogen recovered from patients who have been hospitalized for more than a week. The likelihood of developing a *P. aeruginosa* infection is much greater among colonized patients, and the longer a patient remains in hospital, the greater the change of becoming colonized (Bodey *et al.*, 1983).

In studies regarding the host defence mechanisms against *P. aeruginosa,* emphasis has been placed on the role of antibody-mediated protection. Antibodies to a number of *Pseudomonas* antigens have been identified in patients with *Pseudomonas* infections (Klinger *et al.*, 1978; Pollack and Young, 1979). In spite of the fact that passively transferred antibody has been protective in certain model systems (Pavlovskis *et al.*, 1977), however, the chronic and persistent nature of most *Pseudomonas* infections suggests that this response alone is inadequate.

In studies involving the role of T cells in *Pseudomonas* immunity, it has been demonstrated that this bacterium is capable of eliciting T cell responses in a number of species (Markham *et al.*, 1984; Markham *et al.*, 1985; Parmely *et al.*,

1984; Parmely and Horvat, 1986; Pier and Markham, 1982; Porwell *et al.*, 1983; Powderly *et al.*, 1986). Sorensen *et al.* (1977) originally reported that lymphocytes from patients with cystic fibrosis who have advanced pulmonary disease have low responses to *P. aeruginosa* in vitro. This hyporesponsiveness is both acquired and specific and may play an important role in the irreversibility and destructiveness of the *Pseudomonas* infection.

Later Garzelli *et al.* (1980) and Campa *et al.* (1982) reported that mice with repeated systemic infections caused by *P. aeruginosa* can induce a specific delayed-type hypersensitivity after a challenge in the foot-pad. Furthermore, T lymphocyte-enriched spleen cells from sensitized donors can specifically transfer this delayed-type hypersensitivity to syngeneic recipients. However, the T lymphocyte-enriched spleen cells can not transfer protection against a lethal challenge. This result indicates that in systemic *P. aeruginosa* infections, a dissociation may exist between delayed-type hypersensitivity and acquired cellular resistance.

In other studies, Pier and Markham *et al.* (1982) demonstrated, in a murine model, that Thy-1$^+$ spleen cells from *Pseudomonas*-immune donors have the ability to transfer the protection against a bacterial challenge. They used a high-molecular-weight polysaccharide that was isolated from *P. aeruginosa* culture supernatants and the cytotoxic drug vinblastine, as an antigenic system. Results of further studies by these investigators also suggested a more direct role for T cells in providing protection against *P. aeruginosa*. They describe a Lyt-1$^-$, 2,3$^+$, I-J$^+$

T cell as the final effector cell in the in vitro killing of this extracellular bacterium, and the fact that the T cell killing is mediated by the lymphokine interleukin 1. (Markham *et al.*, 1984; Markham *et al.*, 1985). This was the first indication that T cells may be involved in a role other than that of a helper cell for antibody production in the immunity against *Pseudomonas*.

Results of recent studies in patients with cystic fibrosis by Parmely *et al.* (1984) and Parmely and Horvat (1986) have identified two secreted T cell-stimulating antigens from *P. aeruginosa*. While responses were directed against both elastase and alkaline phosphatase, the majority of the response was towards the latter. Results of these studies provide an unique antigenic system to study the immunity to *Pseudomonas*.

Woods *et al.* (1980) were the first to demonstrate the requirement of pili for adhesion of *P. aeruginosa* to mammalian cells. Using human buccal cells in an adherence assay, these investigators found that preincubation of the buccal cells with purified pili causes a decrease in the adherence of intact *P. aeruginosa* bacteria. This suggests that it is the pili that are responsible for adhesion, since they successfully compete for adherence with whole bacteria.

In our laboratory, we are also concerned with the role of pili in the pathogenicity of *P. aeruginosa*. In particular, we are investigating T cell proliferation responses induced by pili in mice to characterize the nature of cell-mediated immunity against pili in *P. aeruginosa* infections.

T cells from mice immunized with whole bacteria or pili gave good pilus-

specific T cell proliferation responses. These results demonstrate that pili are immunogenic and that even during a whole-bacteria immunization there is a response directed towards the pili. We also tested this response in three different mouse strains to detect any genetic control of this response to pili. All three strains (BALB/c, C57B1/10 and CBA/J) tested produced a T cell proliferative response. Results of studies by Berk and Hazlett (1983) previously demonstrated a murine genetic control of corneal responses to infections caused by *P. aeruginosa*. Certain mouse strains were resistant to infections by *P. aeruginosa;* others were not. Similarly, Markham and Pier (1983) have described a variation in immune response patterns in various mouse strains with regard to their antibody responses to immunization with *P. aeruginosa*. Since we only tested three strains of mice, it is quite possible that there is a genetic control of pilus responsiveness that we have not yet discovered. Studies with other mouse strains are currently under way to evaluate this possibility.

To delineate the T cell antigenic regions of the pilin subunit, T cell blasts were generated from lymph nodes of antigen primed BALB/c mice. These blasts were tested in vitro in T cell proliferation assays for reactivity against the fragments of the pilin subunit prepared by enzymatic digestion. Of the three cT peptides tested, only the cTIII peptide was able to elicit a T cell response. In previous antibody studies, a major antibody binding site was detected in this region as well. In addition, in the previous studies a weakly immunogenic antibody binding site was described in the cTIV region. However, a T cell antigenic site was not

detected in this region in our studies. Like the antibody studies, no response was detected against the cTII region in our T cell studies.

Although we detected a T cell recognition site within the cTIII region, this region is very large (67 residues). Therefore, to pinpoint the antigenic site(s) that may exist within it, we further subdivided it into smaller fragments by enzymatic cleavage. Both the peptides cTIII-T_1 (residues 82-110) and cTIII-T_1-CBII (residues 82-104) were able to stimulate either PAK-specific or cTIII-specific T cell blasts to proliferate (Fig. 4 and 5). None of the other peptides tested produced a response. In previous antibody studies, the region that includes residues 82-110 was identified as the immunodominant site by using anti-pilus antisera. Thus, it appears that both T and B cells recognize this region. In contrast to the results of previous studies (Watts *et al.*, 1983b), no antigenic site was delineated in the region from residues 70 to 81. Previously, this region was identified as a B cell antigenic site that was capable of titrating approximately 20% of the pilus specific antibodies, whereas the region from 82-110 competed with a fraction that represented approximately 60% of the pilus-specific antibodies (Sastry *et al.*, 1985b). Although no detectable T cell reactivity toward this region (residues 70-81) was observed in our studies, it is possible that this peptide, and other smaller peptides tested, do indeed contain T cell epitopes but, because of their size, either cannot be recognized or properly presented by the use of our current experimental design.

Notwithstanding, it does appear that the major T cell epitope in the cTIII peptide is in the region from residues 82 to 110 and that both B and T cell

recognize this region. Common epitopes for B and T cells have been reported for other antigens as well, for example cytochrome *c* (Kilgannon *et al.*, 1986) and ragweed allergen Ra3 (Kurisaki *et al.*, 1986). A peptide that would simultaneously induce both cellular and humoral immunity may prove value in understanding the immunological responses to pili.

Results of previous studies, (Watts *et al.*, 1983b) in which pilus-specific antisera were used, have demonstrated that the two types of *P. aeruginosa* pili, PAK and PAO, show a significant cross-reactivity in both directions. Since the only region of significant homology among PAK and PAO pilins is found at the N-terminus (residues 1-54)(Sastry *et al.*, 1985a), it was concluded that this region is responsible for the cross-reactivity. Furthermore, in previous studies (Watts *et al.*, 1983), of the four PAK cT-peptides, only cTI (residues 1-30) reacted with anti-PAO pilus antiserum.

The results of cross-reactivity studies between PAK and PAO pili at the T cell level indicate that there is significant cross-reactivity in both directions. PAK-immune T cell blasts recognize both and PAO pili, and vice versa. As in the antibody studies, we conclude that the cross-reactivity is due to the N-terminal homology (residues 1 to 54) that exists between the two pili. Since the cTII peptide (residues 31 to 53) was unable to stimulate PAK-immune t cell blasts to proliferate, we postulate that the region responsible for the cross-reactivity is located in residues 1 to 30, the cTI peptide. Further evidence to support this hypothesis was provided by the fact that cTI-immune T cell blasts responded to both PAK and

PAO pili. In comparison, the cTII and cTIV peptides did not produce a T cell proliferation response. Thus, we conclude that a T cell epitope exists in the cTI peptide region as well.

It is of interest that the two pilin subunits (PAK and PAO) share extensive amino acid sequence homology in the cTI region with the pilin subunits from *M. nonliquefaciens, N. gonorrhea,* and *B. nodosus* (Froholm and Sletten 1977, McKern *et al.,* 1983, Schoolnik *et al.,* 1983). The reason postulated for the conservation of this region among the diverse genera is that this region may be involved in a function that is shared by all pili, such as maintaining the polymeric structure of the pili or in subunit-subunit interactions (Sastry *et al.,* 1985b). It is of interest that this particular region is extremely hydrophobic, a property that may aid in subunit-subunit interactions.

We conclude that, for BALB/c mice, there are two major T cell antigenic sites on the PAK pilin molecule. One site is located in the N-terminal portion that encompasses residues 1 to 30. The other site is located at the region of the molecule that encompasses residues 82 to 104. These sites are the same sites as those described by the use of anti-pilus antisera from rabbits. The fact that these sites are not only recognized by two different xenogeneic species (rabbit versus mouse) but by two different arms of the immune system (humoral versus cellular) as well leads us to believe that these are major antigenic sites and are suitable regions for further protective studies with synthetic peptides.

TABLE 2.1. Ability of mice to produce a T cell response against pili

Antigen (concn, μg/well)[a]	Response (mean cpm +/- SD)[b]
Bacterium-induced T cells	
10	39,114 +/- 3,776 (3.1)
20	52,465 +/- 2,221 (4.1)
40	41,022 +/- 1,659 (3.2)
	12,732 +/- 3,255
PAK pili-CFA induced T cells	
10	124,178 +/- 7,739 (17.9)
20	130,328 +/- 11,034 (18.8)
40	140,756 +/- 7,371 (20.3)
	6,921 +/- 200
PPD[c] (40)	34,888 +/- 3,037 (5.0)

[a]T cells and antigen presenting cells were incubated at a dose of 10^6 cells per well with antigen.
[b]The stimulation index is given in parentheses. The stimulation index is mean counts per minute in the experimental group/mean counts per minute with medium alone.
[c]PPD, Purified protein derivative control.

TABLE 2.2 Ability of PAK-immune T cell blasts to respond to various cT peptides[a]

Antigen	Concn (μg)[b]	Response (cpm +/- SD)	SI[c]
None		2,874 +/- 534	
PAK pili	20	69,073 +/- 6,476	24.0
	40	69,352 +/- 5,157	24.1
cTII	20	1,154 +/- 236	
	40	1,276 +/- 278	
cTIII	20	21,810 +/- 1,940	7.6
	40	24,015 +/- 164	8.4
cTIV	20	3,482 +/- 668	
	40	2,963 +/- 517	

[a]T cells and antigen presenting cells were incubated at doses of 10^6 cells per well each.
[b]Concentration of antigen per well.
[c]The stimulation index (SI) is mean counts per minute in the experimental group/mean counts per minute with medium alone.

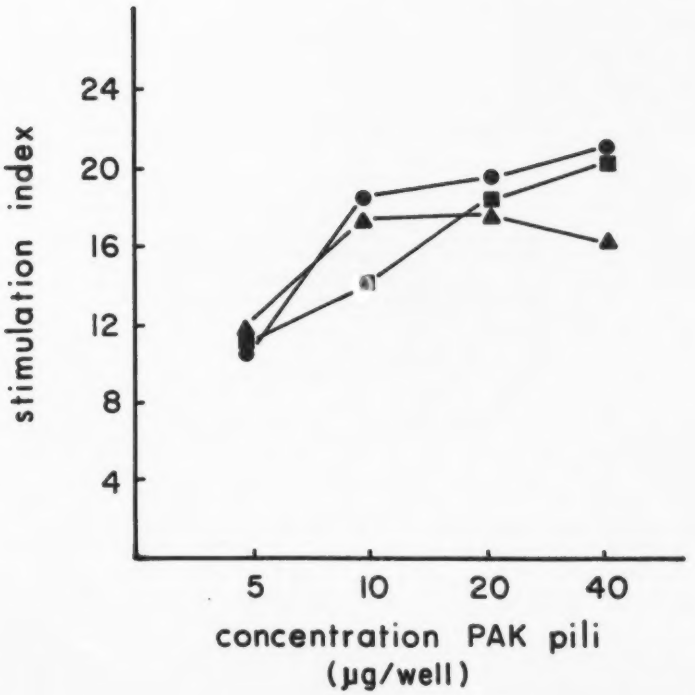

Figure 2.1. Ability of BALB/c (■), CBA/J (▲), and C57B1/10 (●) mouse strains to produce a T cell response against PAK pili. PAK-primed T cells and antigen presenting cells were both used at a dose of 10^6 cells per well. Response is expressed as a stimulation index, which is mean counts per minute in the experimental group/mean counts per minute in the control group. Standard deviations did not differ from the mean by more than 10% (data not shown).

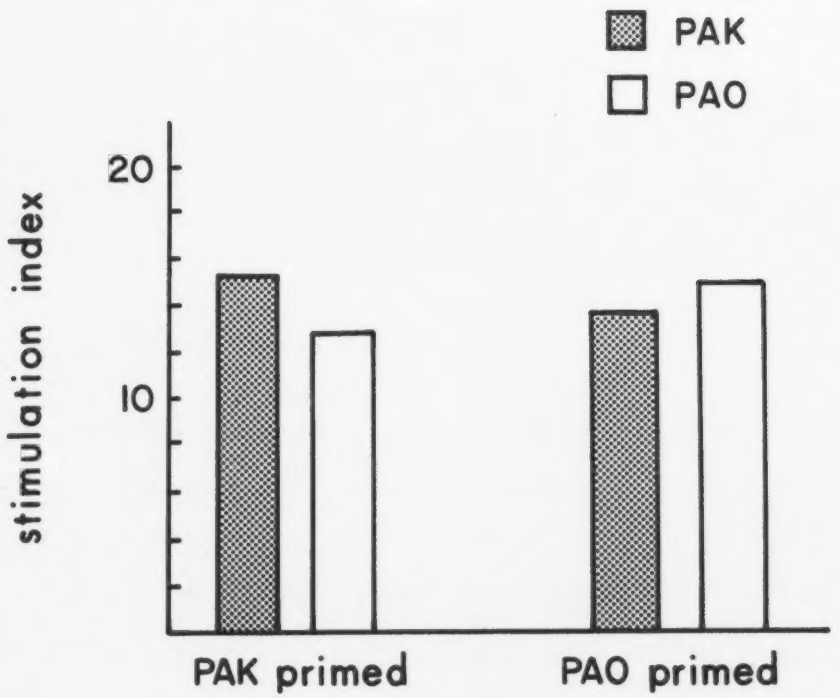

Figure 2.2. Cross-reactivity studies between PAK (hatched) and PAO (open) pili. T cells and antigen presenting cells were both used at a dose of 10^6 cells per well. Response is expressed as a stimulation index (as described in the legend to Figure 2.1) at a chosen antigen dose of 40 μg/well. Standard deviations did not differ from the mean by more than 10% (data not shown).

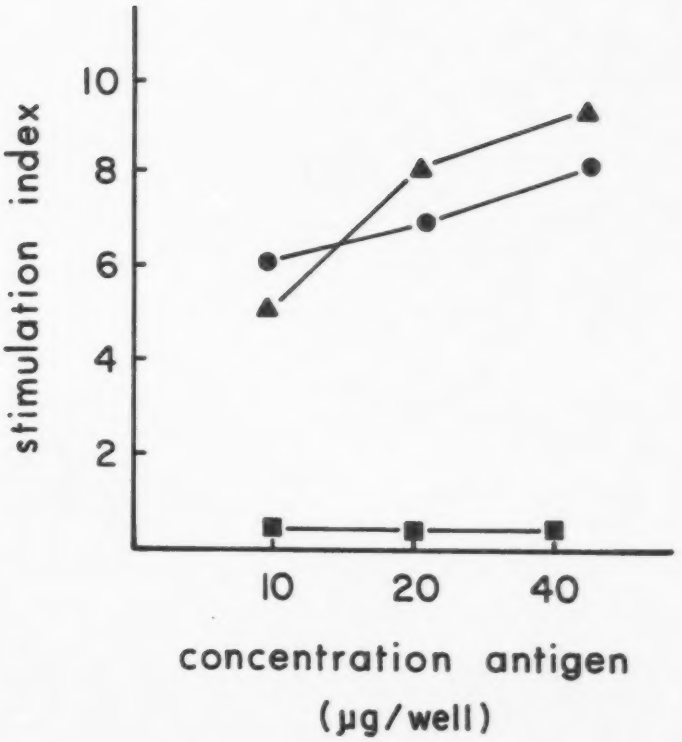

Figure 2.3. Response of cTI-primed T cells to PAK pili (▲), PAO pili (●), and bovine serum albumin (BSA) (■). T cells and antigen presenting cells were both used at a concentration of 10^6 cells per well. Results are expressed as a stimulation index (as described in the legend to Figure 2.1). Standard deviations did not differ from the mean by more than 10% (data not shown).

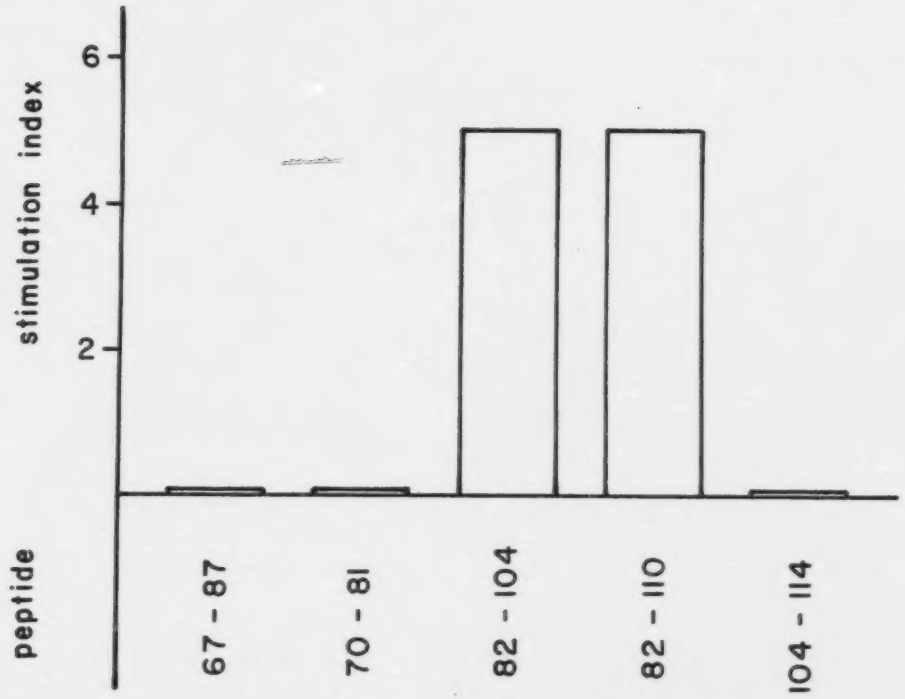

Figure 2.4. Mapping of the antigenic determinant within the cTIII region. The T cells were primed with the cTIII peptide and were used at a concentration of 10^6 cells per well, as were the antigen presenting cells. The concentration of antigen was 40 μg per well throughout. Response is expressed as a stimulation index (as described in the legend to Figure 2.1.). Standard deviations did not differ from the mean by more than 10% (data not shown).

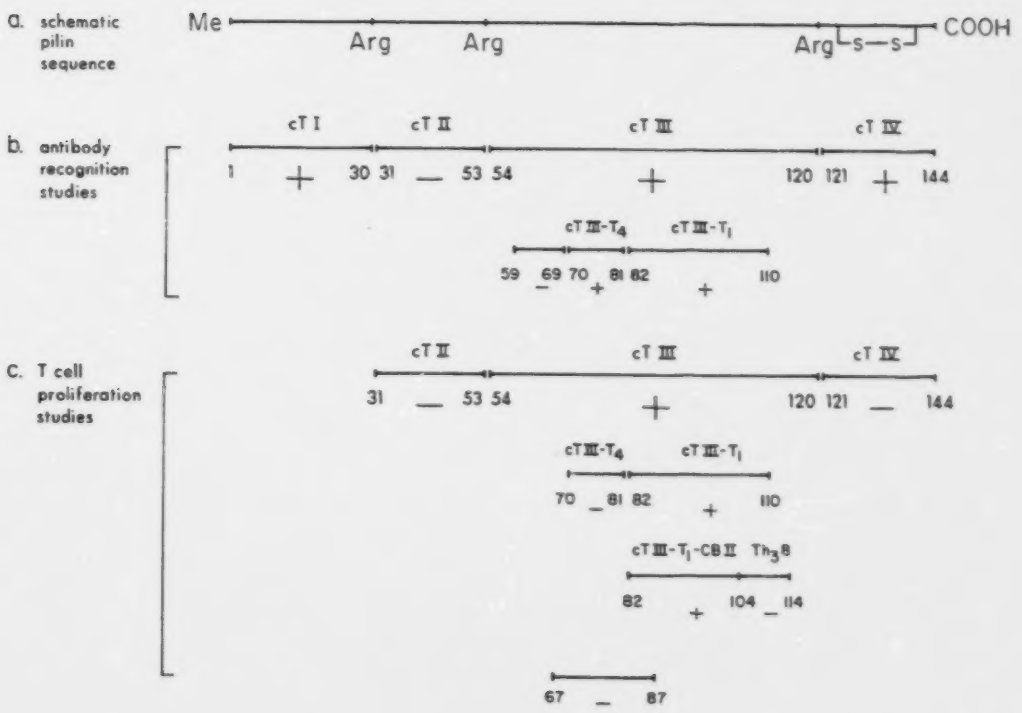

Figure 2.5. Schematic representation of locations of antigenic peptides in *P. aeruginosa* PAK pilin. (A) Schematic representation of the sequence of pilin. The disulphide linkage between residues 129 and 142 is shown. (B) Results of antibody recognition studies, with a map of the cT peptides and a map of the smaller fragments of pilin that have been tested for antigenicity (Watts *et al.*, 1983b). Symbols: +, A positive reaction with anti-PAK pilus antiserum; -, a negative reaction. (C) Results of T cell recognition studies, with a map of the cT peptides and a map of the smaller fragments that were tested for their ability to stimulate PAK-primed T cells to proliferate. Symbols: +, A positive response; -, a negative response.

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CHAPTER III
Immune recognition of polar pili from *Pseudomonas aeruginosa* types K and O[2]

INTRODUCTION

Pseudomonas aeruginosa is an important nosocomial pathogen. It is opportunistic in nature and causes serious infections in those who are immunocompromised. This includes individuals with a disruption in their natural barriers that protect against infection (ie burn victims) and those with weakened immune systems (ie leukemia). In addition, it is also one of the leading causes of morbidity and mortality in those with cystic fibrosis. Antibiotic treatment of those with *P. aeruginosa* infections is often difficult because of the high resistance of this organism to antibiotics. Often a combination of antibiotics is used, and, even then high mortality occurs.

This organism possesses a number of factors which aid it in its virulence. These factors can be divided into those that are secreted exoproducts and those that are components associated with the cell surface. The virulence factors that are secreted include: exotoxin A (Iglewski *et al.*, 1977), exoenzyme S (Iglewski *et al.*, 1978), various proteases such as elastase and alkaline phosphatase (Morihara *et al.*, 1964), and hemolysins such as phospholipase C

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[2]A version of this chapter has been submitted for publication: Smart, W., Sastry, P. A., Paranchych, W., and B. Singh. 1992.

(Johnson *et al.*, 1980; Berk *et al.*, 1987). All of these factors help *P. aeruginosa* resist or interfere with the host's defence system by destroying or degrading various substrates. Some of the virulence factors associated with the cell surface include: lipopolysaccharide (LPS) (Pollack *et al.*, 1979), alginate (or exopolysaccharide) (Woods *et al.*, 1985) and pili (Woods *et al.*, 1980). In our laboratory, we have been concerned with the role of pili in pathogenesis.

P. aeruginosa strains K (PAK) and O (PAO) produce chromosomally encoded polar pili which are flexible filaments with diameters of 5.4 nm and average lengths of 2,500 nm (Weiss *et al.*, 1971; Bradley *et al*, 1972). Each pilus consists of pilin subunits with molecular weights of 15,000 arranged in a helix of five subunits per turn, with each turn having a 4.1-nm pitch (Watts *et al.*, 1983a, Sastry *et al.*, 1985a). *Pseudomonas* pili belong to a class of pili which are present in a wide range of gram-negative bacteria including *Neisseria gonorrhoeae, Neisseria meningitidis, Moraxella bovis, Moraxella nonliquefaciens,* and *Bacteroides nodosus* (Elleman *et al.*, 1984; Froholm *et al.*, 1977; Marrs *et al.*, 1985; Paranchych *et al.*, 1978; Perry *et al.*, 1987; Schoolnik *et al.*, 1984). It has been established that successful colonization of the organism on the mucosal surface is dependent on the presence of pili (Woods *et al.*, 1980). Piliated bacteria that adhere to mammalian cells are often more pathogenic than their nonpiliated counterparts because the pili enable them to become anchored to the host tissue and resist elimination by body fluids.

In recent years, investigators have turned towards developing an

immunoprophylatic approach to *Pseudomonas* infections. An understanding of the immunologic properties of pili may provide the foundation for the development of a synthetic vaccine.

In previous work, Watts *et al.,* (1983b), Sastry *et al.,* (1985b), and Lee *et al.,* (1989) described the B cell antigenic sites on the PAK pili molecule. In addition, we reported T cell immune responses to PAK pili and described the T cell sites on this molecule (Smart *et al.,* 1988). Recently, Lee *et al.,* (1990) have described the B cell antigenic sites on the PAO pilin molecule. In this study, further characterization of the B cell antigenic sites on PAO pilin is reported and, we describe the T cell recognition of PAO pilin. Comparisons are made between the antigenic sites on the K and O pilin molecules and between the T and B cell sites on each molecule. Further, T cell cross-reactivity studies are reported, indicating a cross-reactivity in the cTIII region as well as the previously reported cTI region. In addition, we provide evidence for the requirement of class II major histocompatibility (MHC) antigens and the need for processing of the pili, prior to activation of T cells. Precise information relating to pilus antigenic sites is potentially useful in the development of synthetic vaccines for *Pseudomonas*-related infections.

MATERIALS AND METHODS

Bacteria. The *P. aeruginosa* strains used were PAK/2Pfs and PAO/DB2. Both are multipiliated mutants which were originally obtained from D. E. Bradley, Faculty of Medicine, Memorial University, St. John's, Newfoundland, and have been described previously (Frost and Paranchych, 1977, Paranchych *et al.*, 1979).

Antigens. PAK and PAO pili were purified as described previously (Paranchych *et al.*, 1979).The preparation of the cT_{PAK} and cT_{PAO} peptides (abbreviated cT for citraconylation followed by trypsin digestion) were prepared as described before (Watts *et al.*, 1983b). Briefly, modification of the 12 lysines in pilin was achieved with citraconic anhyride, and this was followed by trypsin digestion of the pilin. This achieves arginine-specific cleavage of the pilin and yielded four convenient fragments, cTI (residues 1 to 30), cTII (residues 31 to 53), cTIII (residues 54 to 120), and cTIV (residues 121 to 144). Initially, the digestion yields both a soluble and an insoluble fraction after centrifugation of the digest. The cT peptides in the soluble fraction (cTII, cTIII, cTIV) were purified by column chromatography on a Sephadex G-50 column and the purity was checked with amino acid analysis, high-voltage paper electrophoresis, N-terminal analysis and sodium dodecyl sulfate-polyacrylamide gels (Watts *et al.*, 1983b). The lysine residues, initially blocked with citraconic anhydride, were deblocked prior

to the use of the peptides as immunogens or in assays. The cT peptides are designated by subscripts corresponding to the pilin molecule they represent.

Animals. Mice of strains BALB/c, C57B1/10J and CBA/J were bred at the Ellerslie Animal Farm of the University of Alberta, or purchased from the Jackson Laboratory, Bar Harbor, Maine. New Zealand White rabbits were bred at the Ellerslie Animal Farm of the University of Alberta.

Preparation of antisera. The antipilus antisera were prepared from New Zealand White rabbits as follows: 100μg of pure pili, suspended in 0.2ml water, was suspended in an equal volume of Freund's complete adjuvant and injected subscapularly and intramuscularly in the gluteal area. The results reported in this paper were obtained with serum from a single bleed of a single rabbit. However, sera from two additional rabbits were also tested and found to contain an almost identical distribution of anti-pilus antibodies. Booster injections were administered at two one month intervals with 100μg of pili suspended in an equal volume of saline and Freund's incomplete adjuvant. The rabbits were bled 2 weeks following the second booster injection.

Fab preparation from rabbit antisera. The IgG fraction of PAO pilus-specific antisera was prepared by affinity chromatography on a protein A-Sepharose column (Pharmacia Fine Chemicals). This fraction was tested for antipilus

activity by ELISA (Enzyme Linked ImmunoSorbent Assay), and then subjected to papain digestion and fragment purification as described previously (Sastry *et al.,* 1985b). A ratio of 1:100 (w/w) of papain to IgG was used in a digestion buffer consisting of 100mM Na_2PO_4 (pH 7.2), 10mM cysteine and 2mM EDTA, with digestion proceeding for 16h at 37°C. Digestion was stopped with 0.15M iodoacetamide, and the digest was immediately loaded onto a Sephadex G-100 column equilibrated with 10mM Tris-HCl (pH 7.2) - 6.7 mM Na_2PO_4 (pH 7.4) - 112 mM NaCl. The column was monitored by a Pharmacia single path monitor UV-1 at 280nm. The Fab fragment was collected and examined for purity by SDS-polyacrylamide gel electrophoresis and tested for anti-PAO pilus activity by ELISA.

Antibody (ELISA) assays. The direct ELISA assay was carried out exactly as described by Worobec *et al.,* (1983). In brief, the appropriate antigens were dissolved in 0.5 M bicarbonate coating buffer (pH 9.6) at a concentration of 5 µg/ml and 0.2-ml aliquots were applied to wells in microtitre plates (Dynatech Laboratories Inc.). After incubation for 16 h at 4°C, the antigen-saturated wells were washed and then allowed to react with appropriate dilutions of either anti-pilus antisera as in the direct ELISA or with antigen-Fab fragments (Fab from IgG isolated from antisera to PAO pili) as in the competitive ELISA. The resulting antigen-antibody complexes were quantitated colorimetrically with goat anti-rabbit IgG conjugated to alkaline Phosphatase (1:2000) purchased from

Boehringer Mannheim Corp., New York, NY. The alkaline phosphatase substrate used was p-nitrophenyl phosphate (Sigma 104 phosphate substrate tablets). Absorbence at 405 nm was determined with a Titertek multiscan ELISA plate reader (Flow Laboratories Inc., McLean, VA).

In the case of the competition ELISA, various concentrations of peptides or whole pili were preincubated with 50 pmol/ml of predominantly pilus-specific Fab in PBS (pH 7.2) and 0.05% Tween 20 in 0.1% BSA for 1 h at 37°C and then overnight at 4°C. Aliquots (200 μl) of each peptide-Fab competition mixture were then transferred to the pilus-coated microtitre wells and allowed to stand at room temperature for 2 h. After washing, the Fab-pilus complexes were quantified colorimetrically as described above. The results are presented graphically as percent inhibition versus competitor concentration. The citraconylated peptides were decitraconylated before use in the competition ELISA.

T cell proliferation assays. These were performed essentially as described earlier (Fotedar *et al.*, 1985). In brief, mice were immunized with 10-25 μg of antigen in equal volumes of CFA per hind foot pad. Popliteal lymph nodes were excised and nylon wool purified lymph node T cells (5×10^5 to 10^6 cells) were incubated, in triplicate, with appropriate doses of antigen and irradiated (3000 rads) syngeneic spleen cells (10^6 cells) for four days in culture medium (RPMI-1640 containing HEPES, glutamine, 5×10^{-5} M 2-mercaptoethanol, 10%

heat-inactivated fetal calf serum and Penicillin-Streptomycin). Incorporation of [^{3}H]thymidine, by pulsing with 1 μCi/well for the final 24 hours, was used as an index of proliferation. Results are expressed as counts per minute (CPM) incorporated or as stimulation index.

T cell lines. T cell lines were generated essentially as described (Kimoto *et al.*, 1980), with minor modifications. Lymph node cells (10^5 cells/ml) from pili/CFA-primed mice were incubated with 50 μg/ml antigen and irradiated (3000 rad) syngeneic spleen cells (2.5 x 10^6 cells/ml) in RPMI 1640 containing HEPES and 10% heat-inactivated horse serum for 8 days. Viable cells were separated by centrifugation on a lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada) cushion. Viable cells obtained from the interface were washed and put back into culture medium (RPMI 1640 containing HEPES, glutamine, 5 x 10^{-5} M 2-mercaptoethanol, 10% heat-inactivated fetal calf serum, and penicillin-streptomycin). T cells (10^5 cells/ml) were cultured for 8 days in the presence of 100 μg/ml of antigen and irradiated (3000 rad) syngeneic spleen cells (2.5 x 10^6 cells/ml). The cycle of separating viable T cells with addition of fresh APC's and antigen was repeated every 8 days. Proliferation assays on the T cell lines were done by incubating 10^4 or 10^5 T cells with 10^6 irradiated (3000 rad) syngeneic spleen cells and various amounts of antigen for 2 to 3 days. Incorporation of [^{3}H]thymidine, by pulsing for 24hr, was used as an index of proliferation.

Monoclonal antibody. The MKD6, anti-IA^d (Kappler *et al.*, 1981) monoclonal antibody was used after ammonium sulfate precipitation of ascites followed by dialysis of the solubilized precipitate. The antibody was sterilized by filtration through a 0.22-μm filter and was used at the dilutions described in the text.

Antigen presenting cells (APC's). For antigen presentation, either irradiated (3000 rad) syngeneic spleen cells (10^6 cells/well) or the $IA^{d/k}/IE^{d/k}$ bearing B cell hybridoma TA3 (Glimcher *et al.*,1982) (10^4 cells/well) were used. For glutaraldehyde fixation, cells were washed twice with Hepes buffered balanced salt solution (HBBSS) and 5 x 10^6 to 10^7 cells/ml in HBBSS were treated with a final concentration of 0.05% of glutaraldehyde (Fisher Scientific), as described (Shimonkevitz *et al.*, 1983). After half a minute at 37°C, fixation was stopped by addition of an equal volume of 0.2 M lysine (Sigma) in HBBSS. Cells were washed twice with HBBSS and resuspended in RPMI medium for assay. The efficacy of glutaraldehyde treatment was determined by [^{3}H]leucine uptake by the fixed cells, following the protocol of Shimonkevitz *et al.* (Shimonkevitz *et al.*, 1983). Maximum uptake by fixed cells was 3% of normal cell uptake.

T cell hybridomas. T cell hybridomas were generated essentially according to established procedures (Kappler *et al.*, 1981), with minor modifications. After an 8-day culture cycle, T cell lines were centrifuged on lympholyte M and viable cells (10^5 cells/ml) were cultured with irradiated (3000 rad) spleen cells (2.5 x

10^6 cells/ml), 100 μg/ml of antigen, and 1% IL 2-containing EL4 supernatant for 4 days. After this time, the viable cells were obtained by centrifugation on lympholyte M, and were fused with the mouse thymoma BW5147 in a 1:1 ratio in the presence of PEG 1000. Hybridomas were selected in the presence of hypoxanthine, thymidine, aminopterin, and ouabain. Mouse red blood cells were used as filler cells. The wells scored positive for growth were assayed for IL 2 production in the presence of antigen and syngeneic spleen cells as described below. Hybridomas selected for antigen/APC-induced IL 2 production were subcloned by limiting dilution. In the initial fusion six hybridomas were selected for further study. All of theses hybridomas were PAK specific (unpublished observations). Hybridoma 11.5.33 was chosen for the studies listed in this paper.

IL 2 assay. T hybridoma cells (10^4 or 10^5) were co-cultured with 10^6 irradiated (3000 rad) spleen cells or 10^4 TA3 cells, in the presence or absence of antigen in 200 μl of culture medium. After 2 days, supernatants were collected and were assayed for IL 2 content in a secondary culture by using CTLL, an IL 2-dependent T cell line (Pierres *et al.*, 1984). The CTLL cells (10^4/well) were cultured for 24 h or 3 days with serial dilutions of primary supernatant (starting at 25% primary supernatant), and the degree of stimulation was measured by the incorporation of [^{3}H]thymidine (1 μCi/well) over 24 h. Results are expressed as CPM incorporated in CTLL cells.

RESULTS

B cell antigenic sites on PAO pilin. In order to obtain quantitative information on the proportion of anti-PAO pilin antibodies in rabbits, directed to various regions on the PAO pilin molecule a competition ELISA was developed. As with the PAK pilin molecule, there are three arginine residues at positions 30, 53, and 120 in the PAO pilin molecule. This conveniently divides the protein into four regions. Therefore, arginine-specific cleavage, as described above and previously performed with the PAK pilin molecule, was used to generate the four PAO fragments: cTI_{PAO} (residues 1 to 30), $cTII_{PAO}$ (residues 31 to 53), $cTIII_{PAO}$ (residues 54 to 120), and $cTIV_{PAO}$ (residues 121 to 144). Again, as with the PAK pilin molecule, the cTI_{PAO} peptide, because of its extremely hydrophobic nature, was largely found in the insoluble pellet obtained after centrifugation of the digest. As a result of its extreme insolubility and tendency to adhere to glass and plastic surfaces, it could not be tested in a quantitative manner.

In these experiments, the antibodies were converted to monovalent Fab fragments (as described above) to avoid complications arising from the cross-linking of adjacent subunits in intact pili by divalent IgG molecules. The competition was performed using the cT_{PAO} peptides in a similar manner to that described by Sastry *et al.*, (1985b) with the PAK pilin molecule. The results of the competition ELISA experiments are shown in Figure 3.1.

It may be seen that the most antigenic cT peptide was $cTIII_{PAO}$, which

reacted with approximately 70% of the pilus-specific Fab antibodies in the preparation. Detectable levels of competition were also observed with the $cTII_{PAO}$ peptide, which reacted with approximately 20% of the anti-pilus antibodies. No competition was detected with $cTIV_{PAO}$. Thus, it is clear from this competition ELISA experiment that $cTIII_{PAO}$ contains the immunodominant epitope for B cells, in the pilus protein. However, although $cTIII_{PAO}$ reacts well with pilus-specific antibodies in the antiserum, its binding constant is approximately 2500-fold weaker than that of intact pili as determined by the molar amounts required to cause 20% inhibition (intact pili, 0.4 pmol/ml; $cTIII_{PAO}$, 1000 pmol/ml) (Figure 3.1). This suggests that the specificity of the epitope in $cTIII_{PAO}$ may be conformation dependent.

Ability of mice to produce T cell responses against *P. aeruginosa* PAO pili. The ability of BALB/c mice to produce a T cell proliferation response to immunizations with PAO pili has been shown by us previously (Smart *et al.*, 1988). BALB/c mice were immunized in the footpad with purified PAO pili, and 10 to 15 days later popliteal lymph nodes were removed and tested in T cell proliferation assays. The results indicated that this strain of mice produce a good anti-PAO pilus T cell response. Furthermore, in the response was cross-reactive with PAK pili Smart *et al.*, 1988).

To determine whether there was a variation in the ability of different mouse strains to respond to *P. aeruginosa* PAO pili, we tested the response of

two additional mouse strains of different MHC haplotypes. The results (Figure 3.2) indicate that these two mouse strains, C57Bl/10 and CBA/J can produce a T cell response to PAO pili. The response of these two mouse strains was comparable to that produced by BALB/c mice to PAO pili. Furthermore, like BALB/c mice, the T cell responses produced by these two strains of mice against PAO pili were cross-reactive with PAK pili (data not shown). Thus, the mouse strains BALB/c, C57Bl/10 and CBA/J all produce T cell responses against PAO and PAK pili and the responses are cross-reactive between the two pili. These mice represent three different H-2 haplotypes, H-2^d (BALB/c), H-2^b (C57Bl/10), and H-2^k (CBA/J). The fact that the responses between the strains was comparable indicates that pili are highly immunogenic at that T cell level in these strains, and that no genetic variability in response was noticed. Further studies were undertaken in the BALB/c mouse strain unless indicated.

Response of T cell blasts to the soluble cT peptides from PAO pilin. The ability of PAO-immune T cell blasts to respond to the three soluble cT_{PAO} peptides was investigated, and the results are given in Table 3.1. The only peptide to elicit a response from the PAO-immune T cell blasts in BALB/c mice was $cTIII_{PAO}$. Neither the $cTII_{PAO}$ nor $cTIV_{PAO}$ peptide was able to stimulate the blasts to proliferate. This observation is similar to that observed with PAK-immune T cells against the soluble cT_{PAK} peptides. In those experiments, only the $cTIII_{PAK}$ peptides was able to stimulate the blasts to proliferate. In addition,

both CBA/J and C57Bl/10J PAO-immune T cell blasts showed similar results to the cT_{PAO} peptides. In those experiments only the $cTIII_{PAO}$ peptide was able to stimulate these strains' PAO-immune T cells to proliferate (data not shown). Thus, for the soluble cT peptides, and in all the strains tested, it appears that only the cTIII peptide contains a T cell site for both PAO and PAK pilin.

T cell cross-reactivity between PAK and PAO pili. Preliminary studies (Smart *et al.,* 1988) demonstrated a cross-reactivity, at the T cell level, between PAK and PAO pili. That is, either PAO- or PAK-immune T cells responded to both PAK and PAO pili. It was postulated that the cTI region (residues 1 to 30) was responsible for this T cell cross-reactivity because of the identical protein sequence, and the fact that this region was reported by previous authors to be responsible for B cell cross-reactivity (Watts *et al.,* 1983). However, direct testing of this hypothesis could not be performed because of the insolubility of this peptide. Indirect evidence was provided by immunizing with the cTI peptide and showing a response of cTI-immune T cells to both PAK and PAO pili (Smart *et al.,* 1988). To determine if other region(s) of the pilin molecule were also responsible for T cell cross-reactivity the response of pilus-immune T cells was tested against the soluble cT peptides of both PAK and PAO pili. Both PAK- and PAO-immune T cell blasts responded to their own and each others cTIII peptides (Table 3.2). There was no response to their own or each others cTII or cTIV peptides. The response to each others cTIII peptides was

somewhat surprising because of the sequence variability between the two pilin proteins in the cTIII region (only approximately 25% homology).

Further support for this cross-reactivity was provided by immunizing mice with the $cTIII_{PAK}$ peptide and testing the resulting $cTIII_{PAK}$-immune T cell blasts against PAK and PAO pili. The $cTIII_{PAK}$-immune T cells were able to respond to both pilus molecules, although the response to the PAK pilus molecule was greater (Table 3.2). Thus, at the T cell level, it appears there is a cross-reactivity between PAK and PAO pili in the cTIII region (residues 54 to 120) as well as the cTI region.

Blocking of T cell response to pili with anti-IA^d antibody. T cells recognize antigen when presented by the self-class II major histocompatibility (MHC) antigens as peptide fragments. Therefore, in understanding the T cell response to various antigens, it is not enough to just identify the T cell epitopes. Complete understanding involves the knowledge of the role of the MHC antigens and the subtype of T cell responding. We investigated by testing the ability of the monoclonal antibody MKD6 (anti-IA^d) to block the response of BALB/c PAK-immune T cells to PAK pili. The results in Table 3.3 clearly indicate that the addition of the MKD6 antibody abolishes the response of BALB/c PAK-immune T cells to PAK pili. Thus, in BALB/c mice the T cell response to pili occurs in the context of IA^d class II MHC molecules. No blocking was seen by other appropriate control antibodies (data not shown).

Further, blocking of the T cell response was also demonstrated with the antibody GK1.5, which is an anti-CD-4 antibody, but not by anti-CD-8 antibody (Frohlich *et al.*, unpublished observations). Thus, the subset of T cells producing a response against pili is of the CD-4$^+$ or helper phenotype and recognizes antigen in the context of Ia molecules.

The requirement of antigen processing for activation of PAK-specific T cell hybridomas. The requirement for antigen processing of PAK pili prior to presentation to T cells was investigated using the method of glutaraldehyde fixation of antigen presenting cells. The antigen presenting cells used were the $IA^{d/k}/IE^{d/k}$ bearing B cell hybridoma TA3. Because TA3 cells cannot be used in T cell proliferation assays, T cell hybridomas were produced as described in Materials and Methods. Glutaraldehyde fixation has been shown to interfere with the normal mechanism of antigen processing. That is: the internalization, degradation and re-expression on the surface, of the antigenic fragments of the molecule. Glutaraldehyde fixed APC's can effectively present smaller antigens, which do not require processing, to T cells for activation. However, larger antigens, requiring the processing steps, are not able to be presented by glutaraldehyde fixed APC's (Shimonkevitz *et al.*, 1983).

TA3 cells were fixed with glutaraldehyde and used to present PAK pili to the PAK-specific T cell hybridoma 11.5.33. This PAK-specific T cell hybridoma is specific for PAK pili (Smart *et al.*, unpublished observations). Fixed TA3 cells

were unable to present pili, whereas normal TA3 cells were still able to process and present pili to 11.5.33 (Table 3.4). This indicated the need for antigen processing of the PAK pili prior to its presentation to T cells. Thus, there is a requirement for processing of the pili, by antigen presenting cells, prior to its presentation by class II MHC molecules, to helper (CD-4+) T cells.

DISCUSSION

It has been established that successful colonization of *P. aeruginosa* on the mucosal surface is dependent on the presence of pili (Woods *et al.*, 1980). Piliated *Pseudomonas* strains that adhere to mammalian cells are often more pathogenic than their nonpiliated counterparts because the pili enable them to become anchored to the host tissue and resist elimination by body fluids. An understanding of the immunological properties of pili may provide information for the development of synthetic vaccines against *P. aeruginosa* pili, which would either prevent attachment, or aid in the elimination of this organism from the body.

The pili from *P. aeruginosa* PAK and PAO consist of protein subunits called pilin. The pilin from both contain 144 amino acids each and neither protein contains phosphate or carbohydrate residues. Sastry *et al.*, (1985a) have compared the amino acid sequences between PAK and PAO pilin. The PAK and PAO pilin molecules share approximately 49% homology between their amino acid sequences. However, this homology differs throughout various regions of the pilin molecules. The N-terminal portions of the two proteins, from residues 1 to 54, are almost identical. In contrast, the major area of amino acid differences is located in the central part of the sequence (residues 55 to 97), where the homology is only approximately 25%. The C-terminal part of the pilin molecule is semi-conserved with about 58% homology between the two pilin proteins, and they both contain a conserved disulphide bridge.

However, despite being only 49% homologous, secondary structure predictions revealed few significant differences between the two pilin molecules (Sastry *et al.*, 1985b). This is in accordance with the findings of Watts *et al.* (1983a), who have reported that PAO and PAK pilins have identical circular dichroism spectra with an alpha-helical content of approximately 47%.

B cell antigenic sites on PAK and PAO pilin.

The first immunological studies on pili were reported by Watts *et al.*, (1983b). Using direct ELISA and immunoblot techniques, they identified four B cell antigenic sites on PAK pilin. One determinant was found in the cTI_{PAK} peptide (residues 1 to 30), two were in the $cTIII_{PAK}$ peptide (residues 54 to 120) and more specifically involving fragments 70 to 81 and 82 to 110, and one was found in the $cTIV_{PAK}$ peptide (residues 121 to 144). The determinants in $cTIII_{PAK}$ and $cTIV_{PAK}$ were type specific and did not cross-react with anti-serum raised against PAO pili. On the other hand, the antigenic determinant in cTI_{PAK} was found to be common to both PAK and PAO pili, and was concluded to be responsible for the cross-reactivity between these two pili.

Although Watts *et al.*, (1983b) identified four antigenic sites, because the direct ELISA and immunoblot techniques are quantitative in nature, it was not known which of these sites was more or less immunogenic. Further B cell studies on the PAK pilin protein were done by Sastry *et al.*, (1985b) using a competition ELISA procedure which is able to determine the proportion of

antibodies binding to each site. They identified that the two $cTIII_{PAK}$ antigenic sites (fragments 70 to 81 and 82 to 110) where the most immunogenic regions of the PAK pilus protein. The fragment 70 to 81 was able to titrate approximately 20% of the PAK-pilus-specific antibodies, while fragment 82 to 110 competed with a fraction representing approximately 60% of the pilus-specific antibodies. No competition was detected with the $cTIV_{PAK}$ peptide, a region identified by Watts *et al.,* (1983b) as a B cell binding site. Furthermore, they could not detect any competition with cTI_{PAK} or with PAO pili, indicating a low level of antibody specific for the epitope in cTI_{PAK}; another B cell binding site identified by direct ELISA. Thus, while four antigenic sites were identified by direct ELISA, the region from 82 to 110 was the major antigenic site for B cells on the PAK pilin protein as determined by competition ELISA.

Preliminary work by Lee *et al.,* (1990), identified the B cell antigenic sites on PAO pili. They found that anti-PAO antisera reacted with seven synthetic peptides encompassing the following residues: 22 to 33, 41 to 49, 58 to 70, 75 to 84, 105 to 114, 117 to 125, and 128 to 144. However, like the work of Watts *et al.,* (1983), these studies were performed with a direct ELISA technique which does not indicate the proportion of antibodies binding to each site. Therefore, we undertook studies with a competition ELISA technique to determine the B cell immunodominant site on the PAO pilus protein using the cT peptides. Two of the three soluble cT peptides were able to compete with the anti-PAO antiserum. These were the $cTIII_{PAO}$ peptide which bound about

60% of the anti-PAO antisera and $cTII_{PAO}$ which competed with approximately 20%. The $cTIV_{PAO}$ region did not compete. Despite the fact that the $cTIII_{PAO}$ region bound most of the anti-PAO antisera, its binding constant was approximately 2500-fold weaker than the intact pili. This observation was also noticed with the PAK pilin competition experiments. Whereas the $cTIII_{PAK}$ region bound most of the anti-PAK antisera its binding constant was about 125-fold weaker than that of the intact pili. This suggests that a significant loss of secondary structure may occur when both pilin are cleaved into smaller pieces. This loss of secondary structure may be important for antibody binding.

These results are similar to those obtained from the PAK studies in that the most immunodominant site was also located in the $cTIII_{PAK}$ peptide. Whether or not this immunodominant site is also confined to the 82 to 110 region in the PAO pilin as it is in PAK is under further investigation. Nevertheless, for both PAK and PAO pilin, the cTIII region is the major B cell antigenic site.

Differences between the PAK and PAO B cell responses were noted in that a B cell antigenic site was shown to exist in the $cTII_{PAO}$ region, in the competition experiments. This finding was not observed in the PAK competition studies. However, Lee *et al.,* (1988; 1990), using synthetic peptides and a direct ELISA techniques have shown binding of both anti-PAK and anti-PAO antiserum to homologous peptides in this region.

Finally, the only regions of B cell cross-reactivity between PAO and PAK pilin were reported to exist in cTI (Watts *et al.,* 1983b) and a region

encompassing residues 41 to 49 (Lee *et al.,* (1990). No other regions of cross-reactivity were reported.

T cell antigenic sites on PAK and PAO pilin.

Identification of T cell antigenic sites on PAK pilin was previously reported by our laboratory (Smart *et al.,* 1988). T cell antigenic sites were identified in the cTI_{PAK} and $cTIII_{PAK}$ peptides. No T cell sites were identified in the $cTII_{PAK}$ or $cTIV_{PAK}$ regions. Within the $cTIII_{PAK}$ region a T cell antigenic site was localized to the region encompassing residues 82 to 110. Thus, for PAK pilin, the region from residues 82 to 110 is both a B and T cell epitope.

Our results in the present studies indicate that PAO pili is equally immunogenic to PAK pili at the T cell level. T cell responses were observed in three strains of mice of different haplotypes. As with previous T cell studies with PAK pili, no difference was noted between the diverse mouse strains in their ability to respond to PAO pili.

The ability of the soluble cT_{PAO} peptides to stimulate PAO-immune T cell blasts was investigated. Only the $cTIII_{PAO}$ peptide was able to stimulate the PAO-immune T cell blasts to proliferate. Neither the $cTII_{PAO}$ or $cTIV_{PAO}$ peptides produced proliferative responses. These results are identical to those observed with PAK-immune T cells to the soluble cT_{PAK} peptides.

Previous studies with PAK-immune T cells also identified a T cell site within the cTI region. Because the two pilin proteins are identical in this region,

and the fact that cTI_{PAK}-immune T cells were able to respond to both PAK and PAO pili in previous studies, we conclude that a T cell site also exists on the PAO pilin protein in the cTI region. Thus, we conclude that, at the T cell level, the response to PAK and PAO pilin are very similar. Both have T cell sites located in the cTIII region. Whether or not this T cell site is also confined to the 82 to 110 region in the PAO pilin molecule, as it is in the PAK pilin molecule, is under further investigation.

This observation that PAK and PAO pilin share T cell immunodominant sites in the cTIII region may be useful information in the development of synthetic vaccines. The incorporation of this region into a synthetic vaccine would serve a number of purposes. First, since the production of antibodies by B cells is dependent on input from T helper cells, the stimulation of T cells by a vaccine containing a T cell epitope would aid in this production. Second, the production of immunological memory would be induced through T memory cells. Finally, T cells themselves have been shown to act as effector cells against bacteria (Markham *et al.,* 1984; Markham *et al.,* 1985). A vaccine containing the T cell epitope may trigger the response of such T effector cells. Thus, in considering the composition of any synthetic vaccine, the inclusion of this region would be advantageous.

In previous work, the cross-reactivity between the two pilins was concluded to be as a result of the cTI region. In this study we investigated the possibility that other sites might also contribute to the cross-reactivity. PAK- and

PAO-immune T cells were reacted against each others cT peptides. When this type of experiment was performed at the B cell level, using anti-pilus antiserum by Lee *et al.,* (1990) only a region from residues 41 to 49 cross-reacted. However, in our T cell studies, both PAK- and PAO-immune T cells responded to each others cTIII peptides. This observation was confirmed by the fact that $cTIII_{PAK}$-immune T cells responded to both PAK and PAO pili. Thus, at the T cell level, there appears to be a cross-reactivity at the cTIII region as well as in the cTI region. This is somewhat surprising because the two pilin share only 25% homology in the cTIII region. Cross-reactivities are usually the result of similar linear sequence recognition. This is the case with the cTI peptide, which is identical in both PAK and PAO pilin. While there exists short regions of sequence homology between the two pilin proteins in the cTIII region, it is also possible that the T cell cross-reactivity may be the result of the recognition of a similar conformation of the peptides generated from the two proteins after they bind to the antigen groove. This finding is important for consideration of future vaccine development. In addition to those attributes described above, the fact that, at the T cell level, there is cross-reactivity between the two pilins in a region that is also a T cell epitope on both molecules, may prove helpful in designing vaccines which would cross-react over a variety of strains.

Requirements for processing and presentation of pili to T cells.

To further understand the nature of T cell responses to pili, studies were

undertaken to understand the role of class II MHC antigens in the recognition of pili. The blocking experiments using the monoclonal antibody MKD6 (anti-IA^q) indicate that these antigens are crucial in the T cell recognition of PAK pili. This indicates that the PAK-immune T cells are not responding to the PAK pili on its own, but rather, T cells need to have pili presented in the context of class II antigens. This rules out the possibility that pili are mitogenic for T cells. The fact that the subtype of T cells responding to pili are indeed T helper cells was further confirmed by the GK1.5 (anti-CD-4+, or anti-helper T cell) antibody blocking experiments (Frohlich *et al.*, unpublished observations). We conclude that the recognition of PAO pili by PAO-immune T cells also requires class II MHC molecules, because of the similarities in the T cell recognition of these two types of pili.

The final question to be answered in the recognition of pili by T cells was whether or not the pili needed to be processed by antigen presenting cells prior to presentation with class II MHC molecules. Our results show that glutaraldehyde fixed antigen presenting cells were unable to present PAK pili to PAK-specific T cell hybridomas. This indicates a need for PAK pili to be processed prior to recognition by PAK-specific T helper cells, in the context of class II MHC molecules. Because of the similarities between the T cell recognition of PAK and PAO pili, we conclude that PAO pili also needs processing prior to its recognition by PAO-immune T cells.

In conclusion, while other sites on the pilin molecule may be important

for such functions as epithelial cell binding, the fact remains that the cTIII region (and most likely residues 82 to 110) is an important T and B cell recognition site on both PAK and PAO pili. The knowledge of these recognition sites will, hopefully, aid in the development of synthetic peptides, which may be used to vaccinate against *P. aeruginosa* infections.

TABLE 3.1. Ability of PAO-immune T cell blasts to respond to various cT peptides[a]

Antigen in Culture	Concn (μg)[b]	Response (cpm +/- SD)	SI[c]
None		2,727 +/- 486	
PAO pili	10	61,596 +/- 5,237	22.6
	20	72,544 +/- 7,649	26.6
$cTII_{PAO}$	10	3,988 +/- 243	
	20	3,549 +/- 238	
$cTIII_{PAO}$	10	18,011 +/- 2,017	6.6
	20	21,834 +/- 1,135	8.0
$cTIV_{PAO}$	10	3,182 +/- 768	
	20	3,779 +/- 511	

[a]T cells from PAO-immune BALB/c mice and APC's (3000 rad irradiated BALC/c spleen cells) were incubated at 10^6 cells per well each.
[b]Concentration of antigen per well.
[c]The stimulation index (SI) is mean counts per minute in the experimental group/mean counts per minute with medium alone.

TABLE 3.2. Ability of PAK-, PAO-, and $cTIII_{PAK}$-immune T cells to respond to various antigens

Antigen[a]	Response (mean cpm +/- SD)	S.I.[b]
PAK-immune T cells		
no antigen	2,395 +/- 64	
$cTII_{PAO}$	2,278 +/- 136	
cTIIIPAO	11,850 +/- 514	4.9
$cTIV_{PAO}$	2,332 +/- 278	
PAK pili	45,637 +/- 6,347	16.7
PAO-immune T cells		
no antigen	2,727 +/- 122	
$cTII_{PAK}$	3,770 +/- 406	
$cTIII_{PAK}$	23,144 +/- 3,718	8.5
$cTIV_{PAK}$	3,118 +/- 529	
PAO pili	61,596 +/- 5,856	22.6
$cTIII_{PAK}$-immune T cells		
no antigen	6,234 +/- 1,150	
PAK pili	41,758 +/- 6,912	6.7
PAO pili	27,725 +/- 3,418	4.5
$cTIII_{PAK}$	20,956 +/- 1,430	3.4

[a]T cells from antigen-immune BALB/c mice and APC's (3000 rad irradiated BALB/c spleen cells) were incubated at 10^6 cells per well with antigen. Results listed were for a standardized antigen dose of 10 μ/well.

[b]The stimulation index (S.I.) is mean counts per minute in the experimental group/mean counts per minute with medium alone.

TABLE 3.3. Ability of anti-IA^d antibody to block response to PAK pili by PAK-immune T cells.

Cells[1]	Antigen[2]	Antibody[3]	Response (cpm)
T cells + APC's			2,036 +/- 173
T cells + APC's + PAK pili			21,796 +/- 3,747
T cells + APC's + PAK pili + MKD6			946 +/- 34

[1]T cells from PAK-immune BALB/c mice and APC's (3000 rad irradiated BALB/c spleen cells) were used at concentrations of 10^6 cells/well.
[2]PAK pili was added at a dose of 20 μg/well.
[3]Monoclonal antibody MKD6 (αIA^d) was added at a final concentration dilution of 1/20 of the ascites.

TABLE 3.4. Requirement of processing of pili prior to stimulation of PAK-specific T cell hybridomas

T cells[a]	APC's[a]	Antigen[c]	Response (cpm +/- SD)
11.5.33	Normal TA3's	-	13,002 +/- 576
11.5.33	Normal TA3's	PAK pili	203,379 +/- 10,128
11.5.33	fixed TA3's[b]	-	8,384 +/- 713
11.5.33	fixed TA3's	PAK pili	7,955 +/- 137

[a]T cell hybridoma (11.5.33) and TA3 cells were used as described in Materials and Methods.
[b]Fixed TA3 cells were prepared as described in Materials and Methods.
[c]Antigen dose = 20μg/well.

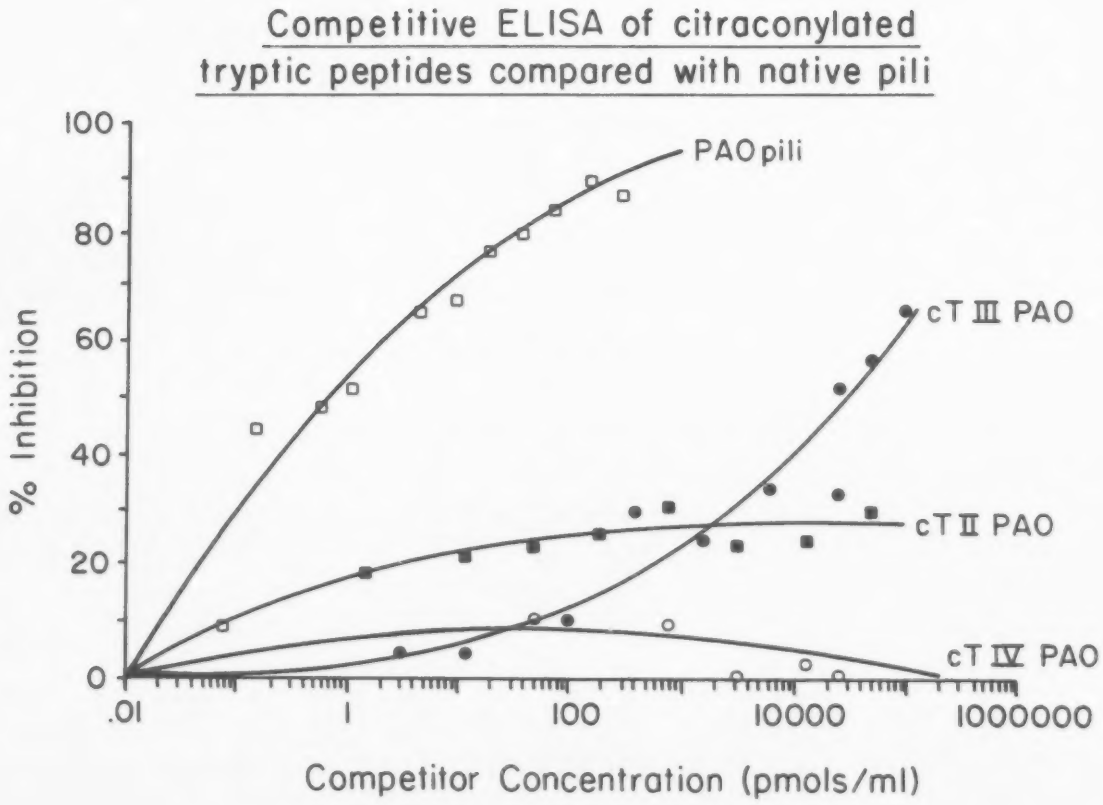

Figure 3.1. Competitive ELISA of cT_{PAO} peptides compared with native PAO pili. Microtitre wells coated with PAO pili were incubated with appropriate dilutions of antigen-Fab fragments. The Fab-antigen complex was quantified using goat anti-rabbit IgG conjugated to alkaline phosphatase. The substrate for alkaline phosphatase was p-nitrophenyl phosphate.

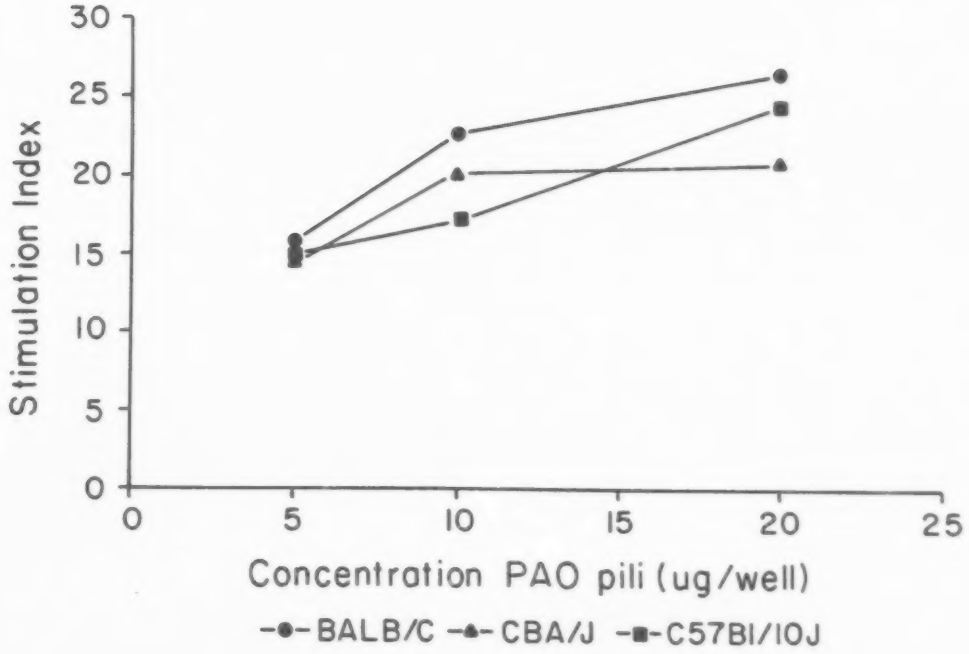

Figure 3.2. Ability of BALB/c (●), CBA/J (▲), and C57B1/10 (■) mouse strains to produce a T cell response against PAO pili. PAO-primed T cells and antigen presenting cells were both used at doses of 10^6 cells per well. Response is expressed as a stimulation index, which is mean counts per minute in the experimental group/mean counts per minute in the control group. Standard deviations which are not shown did not differ from the mean by more than 10%.

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CHAPTER IV
General discussion and future prospects

In this study, we examined the recognition of both PAK and PAO pili from *Pseudomonas aeruginosa* by murine T cells. The principal findings emerging from this analysis are the following:

1. Both PAK and PAO pili are immunogenic and are capable of producing a T cell proliferative response in mice of differing haplotypes. Three different mouse strains were tested (BALB/c - H-2^d, CBA/J - H-2^k, and C57Bl/10 - H-2^k). No significant differences were noted in the immune responses to pili.

2. T cell antigenic sites were found to exist in the cTI and cTIII regions of both pili. In the case of PAK pili, fine specificity mapping defined the region from residues 82 to 110 as the T cell site within the $cTIII_{PAK}$ peptide.

3. T cell cross-reactivity between the two pilins was shown to exist in the cTI and cTIII regions.

4. The requirement for the processing of the pili and presentation by class II MHC antigens was demonstrated prior to T cell recognition.

This is the first study to look at the T cell response to pili. Since pili are an important vehicle for the attachment of an organism to the mucosal surface, and attachment is required for subsequent colonization and infection, a complete understanding of the immune response to pili may provide the information necessary to block or reduce this process. For example, the induction of a T cell response to pili in hosts susceptible to infection may inhibit attachment, mobility and bacterial growth.

The conclusions emerging from this study indicate that T cells are actively involved in the immune recognition of pili from strains K and O of *P. aeruginosa*. The actual T cell recognition sites on both pilin molecules appears to be very similar. In addition, the T cell recognition sites on the pilin molecules are similar to the major B cell recognition sites for these molecules. Knowledge of the T cell sites may prove helpful in the designing of synthetic vaccines against *P. aeruginosa* infections. Vaccines incorporating T cell antigenic sites would be important for a number of reasons. First, since T cell help is required for antibody production, a vaccine incorporating the T cells site(s) would ensure good antibody production. Second, immunological memory would be induced through T memory cells. Finally, the induction of T effector cells, those important in direct T cell killing activities, would be induced.

Furthermore, since T cell cross-reactivity between these two pilins was observed in the same regions as the T cell antigenic sites (ie. cTIII and cTI) perhaps a region could be chosen that would be suitable for inducing T cell

response across a variety of strains. The mapping of T cell antigenic sites on a number of *P. aeruginosa* pili would have to be performed and compared. Alternatively, T cell antigenic sites from different strains could possibly be combined together in a large synthetic carrier and linked with a B cell antigenic site(s) carrier. The goal of either of these approaches would be to induce T cell responses to a variety of *P. aeruginosa* strains.

To accomplish these goals, further specificity mapping within the cTIII region on the PAO pilin molecule is needed. In addition, mapping of those residues responsible for the T cell cross-reactivity between the two pilin molecules is also needed. In addition, as mentioned above, further T cell recognition studies of other *P. aeruginosa* pili are required for the better understanding of the immune response and pathogenicity of *Pseudomonas* infections. Finally, it may also be important to determine the phenotype of the helper T cells induced by *Pseudomonas* pili (such as TH_1 or TH_2 cells). These cells may be important in determining the cytokines produced in an immune response to *Pseudomonas* pili. It is possible that some of the T cells induced may produce cytokines that may be responsible for tissue damage.